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# Phenotypic evaluation of natural and industrial *Saccharomyces* yeasts for different traits desirable in industrial bioethanol production

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**Abstract** *Saccharomyces cerevisiae* is the organism of choice for many food and beverage fermentations because it thrives in high-sugar and high-ethanol conditions. However, the conditions encountered in bioethanol fermentation pose specific challenges, including extremely high sugar and ethanol concentrations, high temperature and the presence of specific toxic compounds. It is generally considered that exploring the natural biodiversity of *Saccharomyces* strains may be an interesting route to find superior bioethanol strains and may also improve our understanding of the challenges faced by yeast cells during bioethanol fermentation. In this study, we phenotypically evaluated a large collection of diverse *Saccharomyces* strains on six selective traits relevant for bioethanol production with increasing stress intensity. Our results demonstrate a remarkably large phenotypic diversity among different *Saccharomyces* species and among *S. cerevisiae* strains from different origins. Currently applied bioethanol strains showed a high tolerance to many of these relevant traits, but several other natural and industrial *S. cerevisiae* strains outcompeted the bioethanol strains for specific traits. These multitolerant strains performed well in fermentation experiments mimicking industrial bioethanol production. Together, our results illustrate the potential of phenotyping the natural biodiversity of yeasts to find superior industrial strains that may be used in bioethanol production or can be used as a basis for further strain improvement through genetic engineering, experimental evolution, or breeding. Additionally, our study provides a basis for new insights into the relationships between tolerance to different stressors.

**Key words** bioethanol, fermentation, high-throughput, phenotype, *Saccharomyces spp.*, stress tolerance

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## Introduction

Taxonomic analysis of the microbiota present in spontaneous alcoholic fermentation processes, such as the fermentation of fruit juice, beer wort, honey or cocoa, revealed a huge yeast diversity, with one yeast species dominating most of the fermentation processes, namely the ascomycetous yeast *Saccharomyces cerevisiae* (Pando Bedriñana et al. 2010; Bokulich et al. 2014; Meersman et al. 2013; Pretorius 2000; Steensels et al. 2014). This dominance has been studied intensively, and it was established that *S. cerevisiae* developed several mechanisms that caused a selective advantage in sugar-rich, alcoholic fermentation-like environments. For example, the Crabtree effect (in which glucose represses respiration) in *S. cerevisiae* is the basis for the so-called "make-accumulate-consume" strategy, where ethanol is first produced to inhibit the growth of other microorganisms, and is later consumed when all fermentable sugars are depleted (Thomson 2005). Furthermore, its ability to grow in both aerobic and anaerobic conditions and its very high glycolytic flux enables it to thrive in all stages of the fermentation process and restrain competing microorganisms (Conant and Wolfe 2007; Goddard 2008; Piškur et al. 2006). Interestingly, the majority of these "fermentative features" emerged not as a response to a man-made fermentation environment, but rather in response to the emergence of angiosperms and fleshy fruits that offered a novel carbon resource worth defending by ethanol accumulation (Thomson et al. 2005; Voordeckers et al. 2012). However, it is well accepted that human intervention has evolved wild *S. cerevisiae* strains into distinct domesticated variants (Diezmann and Dietrich 2009; Fay and Benavides 2005; Steensels and Verstrepen 2014). Examples of traits acquired during domestication include increased copper tolerance (Warringer et al. 2011), flavour production (Hyma et al. 2011) and fructose fermentation capacity (Novo et al. 2009) for wine strains, or an industrially favorable flocculation behavior (Verstrepen et al. 2003) and improved maltose utilization (Voordeckers et al. 2012) in brewing strains. As a result, there are currently several non-domesticated ("wild") and domesticated ("industrial") genetic lineages of *S. cerevisiae* (Liti et al. 2009). These lineages are often not strictly genetically or geographically separated. Indeed, recent genome-wide analysis of the *S. cerevisiae* biodiversity revealed that man-mediated dispersal of this species allowed intercrossing of different lineages generated strains with mosaic genomes that inherited traits from both parental lineages (Diezmann and Dietrich 2009; Legras et al. 2007; Liti et al. 2009). This peculiar genomic structure is not encountered in non-domesticated species, such as the *S. paradoxus*, which is genetically closely related to *S. cerevisiae*.

Compared to e.g. beer, bread or wine fermentations, bioethanol production is a relatively new man-made fermentation process. The bioethanol environment confronts yeasts with new, very specific challenges, which differ greatly from conditions commonly encountered in the traditional

food and beverage fermentations. More specifically, the implementation of very high gravity fermentation with initial sugar concentrations above 300 g $l^{-1}$ to increase cost efficiency requires osmotolerant and ethanol tolerant yeast strains (Pais et al. 2013; Puligundla et al. 2011; Tao et al. 2012; Watanabe et al. 2010). Salt tolerance is important in ethanol production with molasses (Basso et al. 2011). In second-generation bioethanol production with lignocellulose hydrolysates, typical stressors include furfural and 5-hydroxymethylfurfural (5-HMF) originating from the dehydration of pentoses and hexoses, respectively, weak acids formed by the degradation of furfural and 5-HMF (Almeida et al. 2007; Palmqvist and Hahn-Hägerdal 2000, Taylor et al. 2012), acetic acid due to cleavage of acetyl groups from the xylan backbone of the hemicelluloses fraction (Chen et al. 2012), phenolic compounds released due to partial breakdown of lignin (Almeida et al. 2011) and heavy metal contaminants. Lastly, strains are often subjected to thermal stress during the fermentation process, which is caused by the metabolic activity of the yeasts and/or by high environmental temperatures (Basso et al. 2011; Kumar et al. 2013).

Several studies aimed to improve the efficiency of *S. cerevisiae* strains for first- and second-generation bioethanol production by targeting one or few specific traits of interest, such as tolerance to inhibitors or pentose fermentation, e.g. by quantitative trait locus (QTL) analysis, mutagenesis, genome shuffling, metabolic and evolutionary engineering or a combination of these techniques (Demeke et al. 2013; Hubmann et al. 2013; Koppram et al. 2012; Kumari and Pramanik 2012; Pais et al. 2013; Pinel et al. 2011; Shi et al. 2009; Swinnen et al. 2012; Tao et al. 2012). Moreover, several recent studies focus on investigating the potential of wild or other industrial *S. cerevisiae* strains for bioethanol production (Jin et al. 2013; Pereira et al. 2014; Ramos et al. 2013; Wimalasena et al. 2014). Indeed, in the Brazilian bioethanol production with yeast recycling, regular baker's yeast strains initially used as bioethanol fermentations starters were quickly outcompeted by invading wild strains. They dominated and persisted much better in the recycling system than any domesticated yeast strain (Basso et al. 2008; da Silva-Filho et al. 2005). This shows the potential of phenotyping the natural biodiversity of yeasts to find superior industrial bioethanol strains. However, many of these previously performed studies are limited by the number of targeted traits or by the number of studied strains, despite the advent of diverse high-throughput automated phenotypic screening platforms. Nevertheless, such information might be crucial for selection of new, multitolerant strains that can serve as efficient bioethanol production strains or as good candidates for further improvements by, for example, genetic or evolutionary engineering and/or breeding.

In this study, a comprehensive phenotypic evaluation of a large collection of *Saccharomyces* isolates belonging to different species was performed for six stress tolerance traits important for bioethanol production. Studied isolates originated from different habitats and were subjected to

increasing stress intensities in order to map the phenotypic diversity among different *Saccharomyces* species and among *S. cerevisiae* strains from different origins. The most multitolerant strains were selected for fermentation experiments mimicking industrial bioethanol production.

## Materials and methods

*Yeast collection*

A large culture collection, consisting of 373 industrial and wild *Saccharomyces* isolates from diverse origins and belonging to *S. cerevisiae* (279), *S. pastorianus* (46), *S. paradoxus* (38) and *S. bayanus* (10), was used in this study (Table 1). Isolates were identified to the species level by sequencing the variable D1/D2 domain of the large-subunit (26S) ribosomal RNA gene as described by Kurtzman and Robnett (1997). The majority of strains (276) originated from diverse industrial food fermentations, such as ale (122, all *S. cerevisiae*), lager (47, all *S. pastorianus* except one), wine (71, all *S. cerevisiae* except one *S. bayanus*), sake (15, all *S. cerevisiae*), spirit (12, all *S. cerevisiae*) or bakery (9, all *S. cerevisiae*). Additionally, eight *S. cerevisiae* strains that are currently applied in commercial bioethanol production were included. Further, the collection consisted of 82 wild isolates (no *S. pastorianus*) originating from various natural environments, such as oak bark and spontaneous cocoa fermentations, and a number of laboratory yeast strains, including S288c (Mortimer and Johnston 1986) and SK1 (Kane and Roth 1974). All isolates were stored at -80 °C in glycerol based standard storage medium (2% w/v bacto peptone, 1% w/v yeast extract, 25% v/v glycerol) in 96-well microtiter plates. Five strains from different origins were present in each microtiter plate as a control for inter-experiment variation.

*Genotyping*

All isolates were characterized at strain level by interdelta typing according to Legras and Krast (2003), employing the primers delta12 (5'-TCAACAATGGAATCCCAAC-3') and delta21 (5'-CATCTTAACACCGTATATGA-3'). Genomic DNA was extracted as described previously (Meersman et al. 2013). PCR amplification was performed in a reaction volume of 20 µl, containing 200 mM of each dNTP, 1 µM of each primer, 5 units ExTaq polymerase, 1x ExTaq Buffer (Takara, Otsu, Shiga Japan), and 5 ng genomic DNA. Amplification was performed using a C1000 thermal cycler (Biorad, Hercules, California USA) according to the following thermal profile: initial denaturation at 95 °C for 3 min, followed by 9 cycles of 94 °C for 25 s, 45 °C for 30 s and 72 °C for 90 s, and 21 cycles of 94 °C for 25 s, 50 °C for 30 s and 72 °C for 90 s. A final elongation step of 10 min at 72 °C was performed. Amplification products were separated by agarose gel electrophoresis (7 min, 5 kV) on a QIAxcel Advanced System (Qiagen, Venlo, Limburg Netherlands).

*Test conditions and media preparation*

Unless mentioned otherwise, all materials used for media preparation were purchased from Sigma-Aldrich (St. Louis, Missouri USA). All isolates were evaluated for (i) sugar- and/or osmotolerance using increasing concentrations of glucose (ranging from 40% to 70% w/v), fructose (40% - 70% w/v) and sorbitol (30% - 55% w/v), (ii) halotolerance using increasing concentrations of NaCl (500 mM - 3000 mM), KCl (1000 mM - 4000 mM) and LiCl (10 mM - 600 mM), (iii) ethanol tolerance using increasing concentrations of ethanol (5% - 15% v/v), (iv) furan derivative tolerance using increasing concentrations of 5-HMF (2 g $l^{-1}$ - 7 g $l^{-1}$), and (v) metal tolerance using increasing concentrations of $ZnCl_2$ (1 mM – 10 mM), $CuSO_4$ (0.1 mM – 2 mM) and $CdSO_4$ (0.25 mM – 3 mM). Concentrations were selected based on *Saccharomyces* tolerance limits described in literature and can be found in Table S1 (Supplementary Information). For all trait evaluations, YPD agar plates (1.5% w/v agar (Invitrogen, Carlsbad, California USA); 2% w/v bacto peptone (Becton Dickinson, East Rutherford, New Jersey USA); 1% w/v yeast extract (LabM, Heywood, Lancashire UK) and 2% w/v glucose) were used, supplemented with the test compound. Additionally, isolates were incubated on YPD agar without test compound as a control. Further, isolates were tested for thermotolerance (24 °C - 41 °C) on this medium. Carbon and nitrogen sources were autoclaved separately to avoid Maillard reactions, and mixed when the temperature dropped to about 50 °C. Ethanol and 5-HMF were also added after autoclaving.

*Phenotypic screening*

In order to maximize throughput and reproducibility, a high-density array robot (ROTOR HDA, Singer Instruments, Roadwater, Somerset UK) was used to produce and replicate high-density arrays of the yeast collection on the different test media. More specifically, first, the 96-well microtiter plates containing the stored isolates were thawed and spotted on YPD agar and incubated at 30 °C for 48 h. Next, 96-well plates containing 150 µl of standard YPD medium (2% w/v Bacto peptone; 1% w/v yeast extract, and 2% w/v glucose) in each well were inoculated with the isolates using the robot and incubated overnight at 30 °C on a microplate shaking platform (Heidolph, Schwabach, Bavaria Germany) at 900 rpm. In case of screening for ethanol tolerance, isolates were precultured for 48 h in YPD medium with 2% ethanol (v/v) for preconditioning. Then, the optical density at 600 nm ($OD_{600}$) of all wells was measured using a microplate reader (Molecular Devices, Sunnyvale, California USA). Subsequently, cell density was manually adjusted to $OD_{600}\approx0.2$ in a second 96-well microtiter plate using sterile deionized water in order to standardize the starting cell concentration for all isolates. This plate was used as the source plate for spotting the test media. After spotting, all plates were sealed using parafilm and incubated at 30 °C (except for the thermotolerance assays). After five days

of incubation all plates were scanned using a high definition scanner (Seiko Epson, Nagano, Japan). An example of the obtained pictures can be found in Supplementary Material Fig. S2.

*Data analysis*

For each isolate, a phenotypic profile was obtained by assessment of the colony size on the different test media relative to the growth on the control medium (YPD agar), containing no test compounds. For that purpose, scanned images were processed using ImageJ (Abramoff et al. 2004), combined with the ScreenMill software (Dittmar et al. 2010) especially developed for this purpose. Relative growth was calculated as the growth at a certain trait concentration ("test condition") relative to the growth on the control medium. Growth under a test condition is only considered when the relative growth exceeds 5% of the growth on the control medium. The relative growth was further processed by an inverse hyperbolic sine transformation, followed by conversion into Z-scores, which is a statistical measurement of a score's relationship to the mean in a group of scores:

$$Z\text{-}score = \frac{(X - \mu)}{\sigma}$$

with X = transformed relative growth for a particular strain under a particular test condition; μ = mean transformed relative growth for all isolates for a particular test condition; σ = standard deviation of transformed values for all isolates for a particular test condition.

Both genotypic and phenotypic profiles obtained were processed using BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Clustering of the isolates based on genotype was established using a combination of the Dice coefficient (for generating the similarity matrix) and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm. Clustering of the isolates based on phenotype was performed using the Pearson coefficient (for generating the similarity matrix) and the UPGMA algorithm. The equality of overall phenotypic variation among different species and between domesticated and non-domesticated isolates was estimated by calculating the coefficient of variation among isolates at different reference concentrations followed by student's two tailed paired t-test to check for any statistically significant difference ($p$ value < 0.05).

Maximal tolerance limits were defined for each species, and for origin of isolation by the stress concentrations at which at least 10% of the strains showed >5% relative growth. Additionally, for each trait a "reference concentration" was determined (Supplementary Material Table S2) as the concentration where approximately 50% of the investigated isolates managed to grow and showed the highest coefficient of variation. These reference concentrations should therefore be the best concentrations to position isolates in comparison with the whole isolates set. Spearman correlation coefficients were calculated among reference concentrations for each tested trait using SAS software (SAS 9.3, SAS Institute, USA). Further, results were visualized by density plots ("bean plots") using R

for each reference concentration (R Development Core Team, 2013). The pairwise Wilcoxon test for equality of means, combined with the Holm correction for multiple testing, and the Levene's test for equality of variances were performed using R in order to detect significant differences between different isolation origins or between different species. These tests take into account the difference in the number of isolates among groups.

*Fermentation assays*

Seven strains with the best overall performance at high concentrations of glucose, 5-HMF, ethanol and at high temperature were selected and considered multitolerant. For these strains, fermentation experiments at 33% glucose (w/v) were performed to determine their fermentation potential in simulated 1$^{st}$ generation high-gravity bioethanol production medium. Next, the two most promising strains (Y312 and Y232) were selected for fermentations in an artificial medium with 12% w/v glucose and an inhibitor cocktail containing 1.02 g $l^{-1}$ 5-HMF, 0.33 g $l^{-1}$ furfural, 1.9 g $l^{-1}$ acetic acid, 0.36 g $l^{-1}$ formic acid, 0.7 g $l^{-1}$ levulinic acid and 0.033 g $l^{-1}$ vanillin, mimicking the inhibitor concentrations in lignocellulose hydrolysates (Koppram et al. 2012). Similar fermentations were also performed without inhibitors. Fermentation parameters were compared with those of commercial bioethanol production strains (Ethanol Red and CAT1). The $CO_2$ production rate (g $l^{-1}$ $h^{-1}$) was calculated using cubic spline fitting function (Prism 6.04, Graph Pad Software, San Diego California, USA). Lab strain S288c and ale strain Y1 were used as references for strains performing weakly in our high-throughput phenotypic evaluation. Strains were precultured overnight in 3 ml control medium containing 2% glucose at 30 °C. Subsequently, 50 ml YP medium containing 10% glucose was inoculated with the strains at a starting $OD_{600}$ of 0.75 and incubated at 30 °C, 200 rpm for two days until stationary phase. Next, $OD_{600}$ of the precultures was measured and fermentation tubes were inoculated at a final $OD_{600}$ of 2.5 (for 33% glucose) or 0.75 (for artificial fermentation medium mimicking the inhibitor concentrations in lignocellulose hydrolysates) in a total volume of 80 ml. Semi-anaerobic batch fermentation were performed in cylindrical glass tubes with a rubber stopper containing a cotton plugged glass pipe to release $CO_2$. The fermentations were continuously stirred at 120 rpm. This system has been used frequently to mimic the industrial fermentation conditions (Demeke et al. 2013; Hubmann et al. 2013). The weight loss of the tubes due to $CO_2$ release was used to follow the course of fermentation. Fermentations were performed in duplicate. At the end of the fermentation (when the $CO_2$ production rate dropped below 0.01 g $l^{-1}$ $h^{-1}$), 1 ml samples of the fermentation medium were taken, centrifuged and the concentration of ethanol was determined in the supernatant using high performance liquid chromatography (HPLC, Waters isocratic Breeze, ion exchange column WAT010290; Demeke et al. 2013). Column temperature was maintained at 75 °C. 5

mM $H_2SO_4$ was used as eluent with a flow rate of 1 ml $min^{-1}$. A refractive index detector (Waters 2410, Waters, Milford, Massachusetts USA) was used to quantify the compounds of interest.

## Results

*Genetic and phenotypic diversity within the Saccharomyces isolates collection*

Genotyping by PCR-based fingerprinting (interdelta analysis) showed that this collection consists of genetically very diverse isolates. Interestingly, *S. cerevisiae* did not show clearly distinguishable clusters of isolates from different origins (Supplementary Material Fig. S1) except for a separate cluster of ale isolates. Isolates from other origins did not cluster clearly, which is indicative of a broad genetic diversity within the collection. The color gradient in the phenotypic clustering in Fig. 1 shows the tolerance of each *S. cerevisiae* isolate to a given stress concentration, with green being most tolerant and red as least tolerant. The variation in color and color intensities within each column in Fig. 1 demonstrates the vast phenotypic diversity for each of the tested stress concentrations among *S. cerevisiae* isolates. Relative growth of all isolates on all parameters is given in Supplementary Material Table S3. No significant difference in the overall phenotypic diversity was observed between the species *S. paradoxus* and *S. cerevisiae* (student's two tailed paired t-test, $p$=0.62). However, for specific traits the diversity of the *S. paradoxus* isolates was significantly (Levene's test for equality of variances, $p$<0.05) lower than that of the *S. cerevisiae* strains, e.g. under osmostress (39% difference in coefficient of variance), NaCl stress (27%), KCl stress (55%), Cd stress (71%), while the diversity of the *S. cerevisiae* isolates was significantly lower than that of the *S. paradoxus* isolates for LiCl stress (30%), ethanol stress (17%), thermal stress (77%) and Cu stress (49%). Additionally, the overall phenotypic diversity among the non-domesticated (wild) *S. cerevisiae* isolates was found to be 6% less than the domesticated *S. cerevisiae* isolates.

*Comparison of stress tolerance between different Saccharomyces species*

A significant and very high correlation (r=0.78-0.85, Table 3) between tolerance to the three different osmolytes used in this study (glucose, fructose and sorbitol) was observed. Therefore, only osmotolerance in glucose medium will be discussed henceforward. *S. pastorianus* was the least tolerant to all stress factors compared to the other species (Table 1 and Fig. 2) and in particular for osmostress, ethanol stress and 5-HMF stress. *S. paradoxus* generally grew better compared to the other species in high osmolyte concentrations, e.g. 76% of the isolates (29 isolates) managed to grow in 48% glucose (w/v) (reference concentration), compared to only 13% (6 strains) of *S. pastorianus*, 55% (153 isolates) of *S. cerevisiae* and 60% (6 strains) of *S. bayanus* isolates. Similarly, significant differences were noticed in the KCl tolerance assay (Fig. 2 and Table S4A), in which 97% (37) of *S. paradoxus* isolates exhibited growth at the reference concentration compared to 26% (12) of

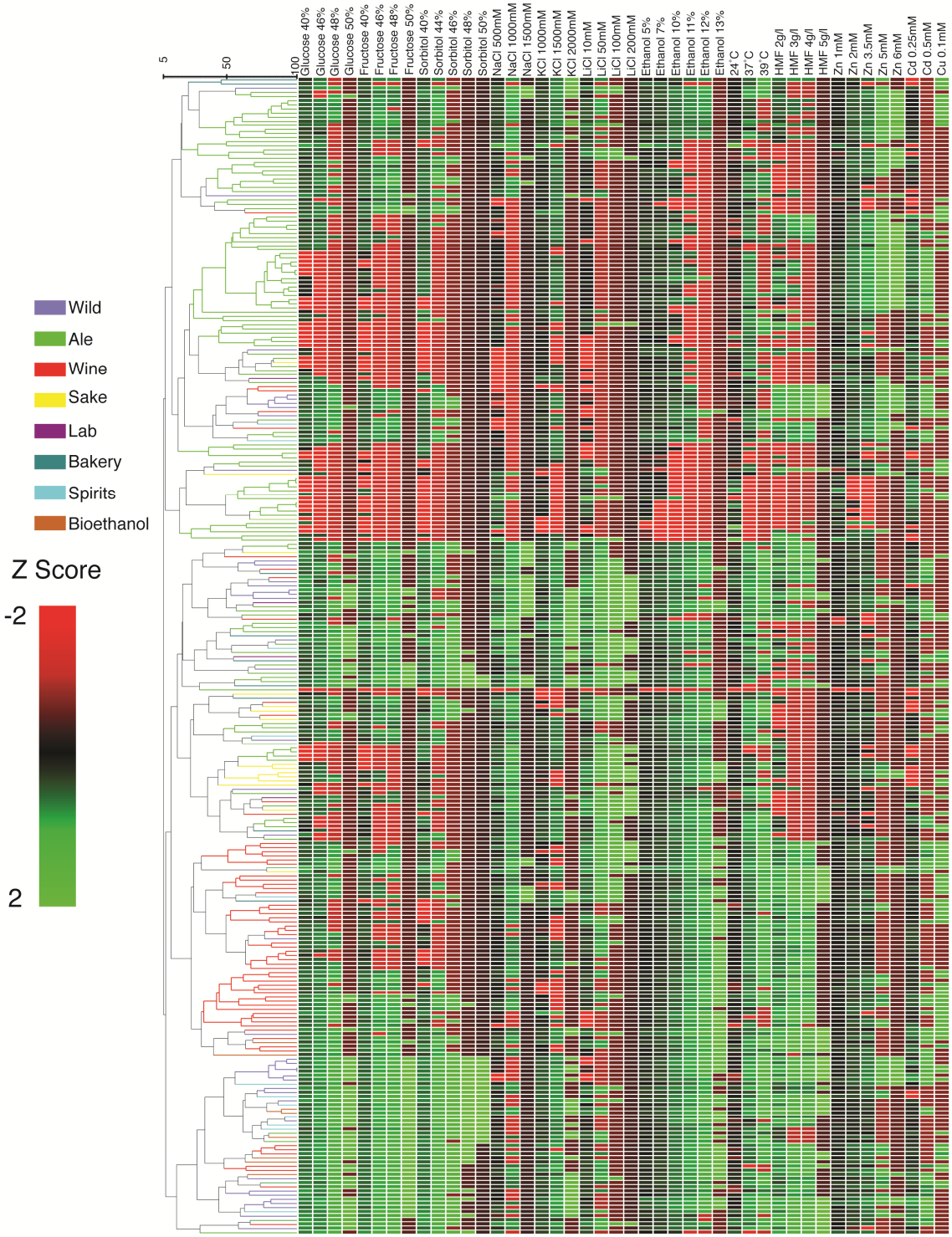


**Fig. 1:** UPGMA phenotypic clustering using the Pearson coefficient of the relative growth of the *Saccharomyces cerevisiae* isolates for all stress conditions investigated in this study. Isolates from different origins are marked in different colors (see figure for color legend). High tolerance is marked as green, while low tolerance is marked with red. The variation in color and color intensities demonstrates the vast phenotypic diversity for each of the tested stress concentrations among *S. cerevisiae* isolates.

*S. pastorianus*, 40% of *S. bayanus* (4) and 75% (209) of *S. cerevisiae*. Interestingly, osmotolerance and KCl tolerance were correlated (r=0.42), while only a weak positive correlation (r=0.14-0.15) was identified with other salt stressors, i.e. NaCl and LiCl, which however were highly correlated with each other(r=0.72) (Table 2). *S. cerevisiae* and *S. bayanus* were both significantly more tolerant to ethanol compared to *S. paradoxus* and *S. pastorianus* (Fig. 2 and Table S4A), with isolates tolerating up to 14% ethanol (v/v), whereas the latter species was limited to 12% ethanol (v/v) (Table 1). On average, 5-HMF tolerance was similar among all species except *S. pastorianus* of which almost all strains were inhibited at the reference concentration (3 g $l^{-1}$). In contrast, even at 4 g $l^{-1}$ 5-HMF, nearly 50% of the *S. cerevisiae* isolates showed growth and 12 isolates managed to grow up to 7 g $l^{-1}$ 5-HMF. In case of metal tolerance, trends were dependent on the metal tested. Tolerance to Zn was similar among species with growth observed up to 6 mM for *S. cerevisiae, S. paradoxus* and *S. pastorianus*. In case of Cd, *S. paradoxus* showed significantly higher tolerance compared to the other species, whereas *S. bayanus* was significantly more tolerant to Cu stress (Fig. 2). Interestingly, at low Zn and Cu concentrations, a large number of isolates showed better growth than on the control plate. For example, at 0.1 mM $CuSO_4$ and 0.1 mM $ZnCl_2$, 77% and 52% of the isolates, respectively, showed better growth compared to the control condition. Finally, *S. cerevisiae* was significantly more thermotolerant than *S. paradoxus* and *S. pastorianus* with 62% of the isolates (173 isolates) growing at 39 °C compared to only 5% (2) and 15% (7), respectively (Fig. 2).

*Comparison of stress tolerance between Saccharomyces cerevisiae isolates from different origins*

In terms of osmotolerance, the majority of isolates showed considerable growth inhibition (on average 70% inhibition of the growth) at 40% glucose (w/v) (the lowest concentration tested). In general, isolates from ale, bakery and sake were less tolerant to high sugar concentrations compared to isolates from other origins (Fig. 3). For example, bioethanol, wine, spirits and wild isolates showed significantly higher osmotolerance at the reference concentration (48% glucose, w/v) compared to ale strains (Fig. 3 and Table S4B). In particular, seven wild isolates and one bioethanol isolate were exceptionally osmotolerant and showed considerable growth up to 55% glucose (w/v) (Table 1).

Wild isolates appeared to be significantly more tolerant to KCl than sake, ale and wine strains (Fig. 3 and Supplementary Material Table S4B). Although not observed for the selected reference concentration (1500 mM KCl; Fig. 3), bakery, bioethanol and spirits strains were also moderately KCl tolerant with nearly 50% of the strains that managed to grow at 2000 mM KCl (respectively 5, 5 and 4 strains). Isolates from different origins were on average equally tolerant to LiCl except for sake strains that were significantly more tolerant compared to ale strains (Fig. 3). In addition, bakery strains showed the highest tolerance to LiCl with on average 78% of the isolates (7 strains) that managed to grow in the presence of 100 mM LiCl.

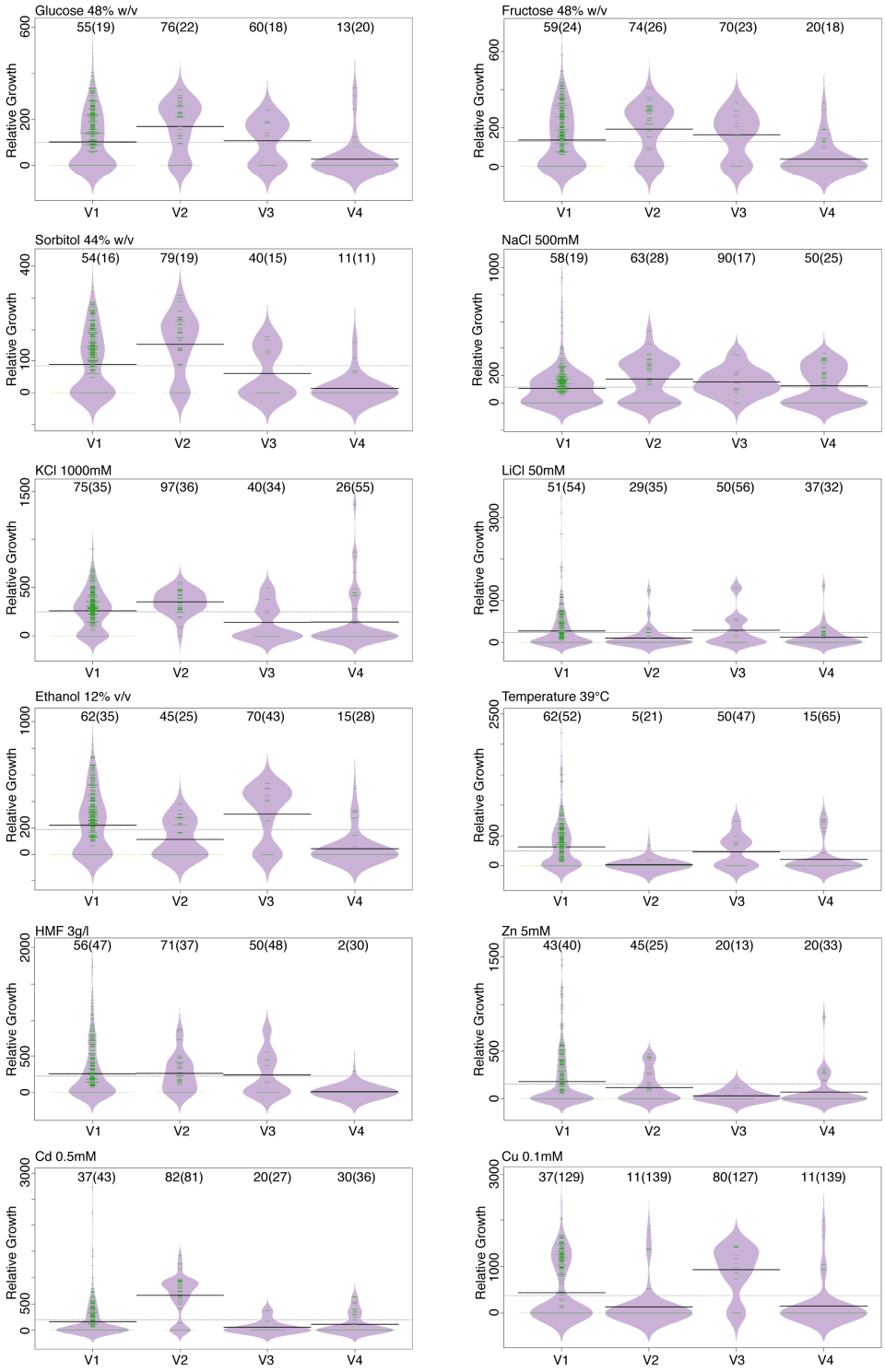


**Fig. 2:** Beanplots illustrating the relative growth (Y-axis) of strains of different *Saccharomyces* species in our collection under reference concentrations for each trait with V1: *S. cerevisiae*, V2: *S. paradoxus*, V3: *S. bayanus*, V4: *S. pastorianus*. The black line represents the average relative growth for each group, the dotted line that among all groups. The shape of the bean represents the distribution. Above each plot, the percentage of the number of strains growing at the reference concentrations with relative growth >5% is indicated and within bracket the average relative growth (%) of strains growing in the presence of the reference concentrations.

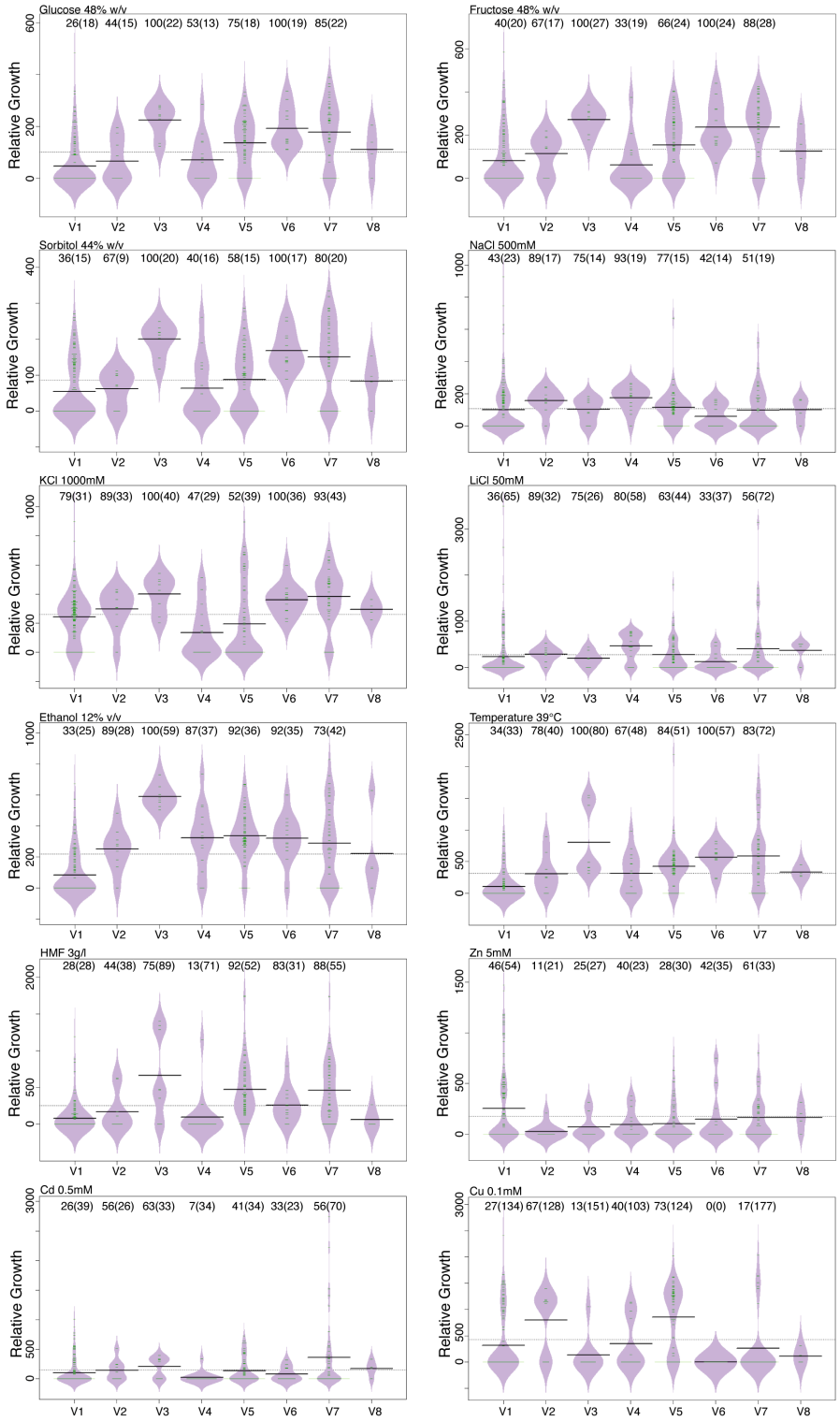


**Fig. 3:** Beanplots illustrating the relative growth of *Saccharomyces cerevisiae* strains coming from different origins in the presence of the reference concentrations, with V1: Ale, V2: Bakery, V3: Bioethanol, V4: Sake, V5: Wine, V6: Spirit, V7: Wild, V8: Lab. Above each plot, the percentage of the number of strains growing in the presence of the reference concentrations with relative growth >5% is indicated and within brackets the average relative growth of strains growing in the presence of the reference concentrations.

**Table 1:** Overview of the number of isolates and tolerance limits[(a)] of different *Saccharomyces* species and for different origins of *S. cerevisiae* isolates in this study.

| | No of isolates | Glucose w/v% | Fructose w/v% | Sorbitol w/v% | NaCl mM | KCl mM | LiCl mM | Ethanol v/v% | Temp °C | HMF g $l^{-1}$ | $ZnCl_2$ mM | $CdSO_4$ mM | $CuSO_4$ mM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | | | | | | | | | | | | | |
| *S. cerevisiae* | 279 | 50 | 50 | 48 | 1500 | 2000 | 200 | 14 | 39 | 5 | 6 | 0.5 | 0.2 |
| *S. paradoxus* | 38 | 50 | 50 | 50 | 1000 | 2000 | 100 | 12 | 37 | 5 | 6 | 1 | 0.1 |
| *S. pastorianus* | 46 | 50 | 48 | 44 | 1500 | 1500 | 100 | 12 | 39 | 2 | 6 | 0.5 | 0.1 |
| *S. bayanus* | 10 | 48 | 48 | 46 | 1500 | 1500 | 200 | 14 | 39 | 5 | 5 | 0.5 | 0.1 |
| **Origin of *S. cerevisiae* isolates** | | | | | | | | | | | | | |
| Ale | 122 | 48 | 48 | 46 | 1000 | 2000 | 200 | 12 | 39 | 4 | 7 | 0.5 | 0.1 |
| Wild | 41 | 55 | 55 | 50 | 1500 | 2000 | 200 | 13 | 39 | 7 | 6 | 0.5 | 0.1 |
| Wine | 64 | 50 | 50 | 46 | 1500 | 1500 | 200 | 14 | 39 | 5 | 5 | 0.5 | 0.2 |
| Sake | 15 | 48 | 48 | 48 | 1000 | 1500 | 200 | 14 | 39 | 4 | 6 | 0.25 | 0.2 |
| Spirits | 12 | 50 | 50 | 50 | 1000 | 2000 | 100 | 13 | 39 | 5 | 6 | 0.5 | <0.1 |
| Bakery | 9 | 50 | 50 | 50 | 1500 | 2000 | 200 | 12 | 39 | 5 | 5 | 0.5 | 0.2 |
| Bioethanol | 8 | 55 | 55 | 50 | 1000 | 2000 | 50 | 14 | 39 | 6 | 6 | 0.5 | 0.1 |
| **Control and Industrial strains profiles** | | | | | | | | | | | | | |
| S288C | | 48 | 48 | 44 | 1000 | 2000 | 200 | 12 | 39 | <2 | 3.5 | 0.25 | 0.1 |
| SK1 | | 46 | <40 | 40 | <500 | 1500 | <10 | 10 | 39 | <2 | 5 | 0.5 | <0.1 |
| Ethanol Red | | 50 | 50 | 50 | 1000 | 2000 | 50 | 14 | 39 | 5 | 3.5 | 0.5 | <0.1 |
| CAT1 | | 50 | 55 | 50 | 1000 | 2000 | 50 | 13 | 39 | 2 | 3.5 | 0.25 | <0.1 |

[(a)] Tolerance limit is defined as the tested concentration of each trait at which at least 10% of the isolates exhibited >5% growth.

**Table 3:** Overview of the tolerance limit, growth (at reference concentrations) and fermentation parameters in 33% glucose of the selected multitolerant strains obtained in this study, two industrial bioethanol strains and two selected non-multitolerant strains.

| Strain name | Origin | Osmotolerance | | Ethanol Tolerance | | HMF Tolerance | | Thermotolerance | | Fermentation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Tolerance limit (%) | Growth at 48% Glucose (%) | Tolerance limit (%) | Growth at 12% EtOH(%) | Tolerance limit (g $l^{-1}$) | Growth at 3 g $l^{-1}$ HMF (%) | Tolerance limit (°C) | Growth at 39°C (%) | Theoretical Yield (%) | Time to reach Vmax (h) | C50 (h)[(a)] |
| Y745 | Wild | 50 | 32.4 | 14 | 63.5 | 7 | 66.2 | 41 | 116.9 | 83 | 18.33 | 40.51 |
| Y313 | Wild | 50 | 24.5 | 12 | 25.9 | 7 | 91.7 | 39 | 85.3 | 86 | 20.26 | 40.51 |
| Y314 | Wild | 50 | 22.4 | 13 | 61.6 | 7 | 77.4 | 39 | 161.5 | 83 | 24.12 | 40.51 |
| Y312 | Wild | 50 | 25.5 | 13 | 59.9 | 7 | 75.1 | 39 | 158.6 | 84 | 22.19 | 38.58 |
| Y310 | Wine | 50 | 21.7 | 14 | 45.6 | < 2 | 0 | 39 | 95.3 | 86 | 18.33 | 36.65 |
| Y224 | Wine | 50 | 28.4 | 14 | 52.3 | 4 | 36 | 39 | 43.8 | 85 | 20.26 | 32.79 |
| Y232 | Wine | 50 | 28.1 | 14 | 51.7 | 4 | 66.8 | 37 | 0 | 85 | 18.33 | 30.86 |
| CAT1 | Bioethanol | 50 | 27.6 | 13 | 56.8 | 2 | 0 | 39 | 40.6 | 88 | 18.33 | 30.86 |
| Ethanol Red | Bioethanol | 50 | 24.1 | 14 | 21.9 | 5 | 128.7 | 39 | 150.3 | 91 | 18.33 | 32.79 |
| s288c | Lab | 48 | 9.4 | 12 | 12.5 | < 2 | 0 | 39 | 27.2 | 78 | 18.33 | 63.67 |
| Y1 | Ale | < 40 | 0 | 10 | 0 | < 2 | 0 | 39 | 38.5 | 61 | 31.83 | 79.1 |

[(a)] C50: Time to consume 50% of the initial sugar content

Ale strains were significantly less tolerant to 12% ethanol (v/v), while bioethanol strains performed significantly better than the average ethanol tolerance levels of isolates from other origins (Fig. 3). Isolates from all other origins showed no significant difference in ethanol tolerance, however, a large variation of tolerance is observed within each origin, especially for wild isolates (Fig. 3). Some wild isolates even managed to grow at very high ethanol concentrations (14%). On average, ale strains were also significantly less tolerant to 5-HMF compared to bioethanol, wine, spirit and wild isolates (Fig. 3). A large variation was seen among wild isolates (Fig. 3). Most wine, bioethanol and wild isolates were still able to grow at 4 g $l^{-1}$ 5-HMF (91% (58), 88% (7) and 83% (34) respectively). Twelve isolates, of which eight were wild isolates, managed to grow even at 7 g $l^{-1}$ 5-HMF.

In order to determine metal tolerance three metals were selected, i.e. Zn, Cd and Cu. Results show that no correlation (Table 2) exists between tolerance to Cu and the two other metals (r=0.02-0.04), whereas a weak correlation was observed between tolerance to Cd and Zn (r=0.29). On average, no significant difference in tolerance to the reference concentration of Zn was observed among all origins (Fig. 3). Nevertheless, ale, wild and sake isolates represented most of the isolates (89%) still able to grow at 6 mM and four ale strains were able to grow up to 8 mM. Similarly, no significant differences in average tolerance to Cd were observed among the isolates from different origins. From 1 mM onwards, growth of all, except five wild isolates, was inhibited. We identified two *S. cerevisiae* wild isolates that can tolerate up to 2 mM of $CdSO_4$. Results showed that wine strains were most tolerant to 0.1 mM of Cu with 73% (47) of the strains that managed to grow and showed significantly higher Cu tolerance compared to ale, sake, spirit and wild isolates (Fig. 3). Surprisingly, 67% (6) of the bakery strains also showed considerably high Cu tolerance. We identified 12 *S. cerevisiae* strains that managed to grow up to 0.3 mM Cu concentration, from which six originate from the winery environment.

**Table 2**: Correlations[(a)] between different reference concentrations[(b)] for the *S. cerevisiae* isolates.

| | Glucose 48% | Fructose 48% | Sorbitol 44% | NaCl 1000 mM | KCl 1500 mM | LiCl 50 mM | ET12% | T 39°C | HMF 3 g $l^{-1}$ | $ZnCl_2$ 5mM | $CdSO_4$ 0.5mM | $CuSO_4$ 0.1mM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glucose 48% | | 0.84 | 0.78 | 0.14 | 0.42 | 0.15 | 0.56 | 0.41 | 0.47 | -0.01 | 0.22 | 0.10 |
| Fructose 48% | | | 0.85 | 0.18 | 0.52 | 0.13 | 0.47 | 0.38 | 0.37 | 0.00 | 0.16 | 0.06 |
| Sorbitol 44% | | | | 0.14 | 0.52 | 0.10 | 0.45 | 0.38 | 0.34 | 0.08 | 0.19 | 0.02 |
| NaCl 1000 mM | | | | | 0.21 | 0.72 | 0.31 | 0.27 | -0.01 | -0.23 | -0.09 | 0.11 |
| KCl 1500 mM | | | | | | 0.22 | 0.34 | 0.30 | 0.21 | 0.07 | 0.16 | -0.09 |
| LiCl 50 mM | | | | | | | 0.34 | 0.30 | 0.05 | -0.32 | -0.08 | 0.05 |
| ET12% | | | | | | | | 0.58 | 0.40 | -0.16 | 0.18 | 0.21 |
| T 39°C | | | | | | | | | 0.36 | -0.16 | 0.21 | 0.08 |
| HMF 3 g $l^{-1}$ | | | | | | | | | | 0.05 | 0.40 | 0.18 |
| $ZnCl_2$ 5mM | | | | | | | | | | | 0.29 | 0.02 |
| $CdSO_4$ 0.5mM | | | | | | | | | | | | 0.04 |
| $CuSO_4$ 0.1mM | | | | | | | | | | | | |

[(a)] All significant trait correlations ($p$ <0.05) are indicated with black font color. Correlations that are not significant are indicated with red font color. Cells with larger correlation values are colored blue, while cells with smaller values are colored green to yellow. Cells with the smallest value are colored red.

[(b)] concentration at which approximately 50% of the investigated strains managed to grow and showed the highest coefficient of variation (Supplementary Material Table S2).

*Potential of selected multitolerant Saccharomyces cerevisiae strains in first and second-generation bioethanol fermentations*

To validate the potential of the obtained dataset for selection of industrially applicable *Saccharomyces* strains, two proof-of-principle experiments, aiming at the identification of strains suitable for first and/or second generation bioethanol production were conducted. Fermentation assays in simulated 1$^{st}$ generation high gravity bioethanol production medium allowed us to compare the ethanol productivity and fermentation efficiency of the seven selected multitolerant strains (Table 3). Ethanol Red yielded most ethanol and produced 91% of the theoretical ethanol yield, followed by CAT1 which produced 88% of the theoretical yield. The ethanol yields of the selected multitolerant strains were comparable (83%-86% of the theoretical ethanol yield). However, strains Y312 and Y232 were the fastest in consuming 50% of the starting sugar concentration among the tested wild and wine strains, respectively (Table 3). These two strains were therefore subjected to a fermentation mimicking a second-generation bioethanol fermentation of lignocellulose hydrolysate containing multiple inhibitors and 12% glucose (w/v) (Fig. 4A). In these conditions, the industrial bioethanol strain CAT1 needed nearly 47 h to consume 50% of the sugars, while Ethanol Red and Y232 were significantly faster (23 h and 25 h, respectively). Strain Y312 was also significantly faster than CAT1, but slower than Ethanol Red and Y232. The HPLC analysis after completion of the fermentation indicated that the ethanol yield was nearly 100% of the theoretical yield for all four strains (Table 4). No significant differences were found between these strains in terms of fermentation performance in 12% glucose without inhibitors (Fig. 4B).

## Discussion

In this study, a broad collection of genetically diverse *Saccharomyces* yeasts, belonging to four different species (namely *S. cerevisiae*, *S. paradoxus*, *S. pastorianus*, and *S. bayanus*) and originating from different industrial and natural habitats, was phenotyped on six selective traits relevant for bioethanol production, including osmotic, thermal, ethanol, salt, heavy metal and 5-HMF stress. Our results demonstrate a remarkably large phenotypic diversity among different *Saccharomyces* species and among *S. cerevisiae* isolates from different origins, extending the results of Kvitek et al. (2008), Skelly et al. (2013), and Warringer et al. (2011).

Hyperosmotic stress tolerance of *S. cerevisiae* and the molecular basis behind it has been investigated extensively. In hyperosmostress tolerance studies, *Saccharomyces* yeasts have been generally subjected to hypertonic growth conditions using glucose or sorbitol as the solute (Tokuoka 1993; Tedrick et al. 2004; Dudley et al. 2005; Watanabe et al. 2010). Additionally, in a few cases, osmostress tolerance has also been evaluated using NaCl or KCl as the solute (Babazadeh et al. 2013;

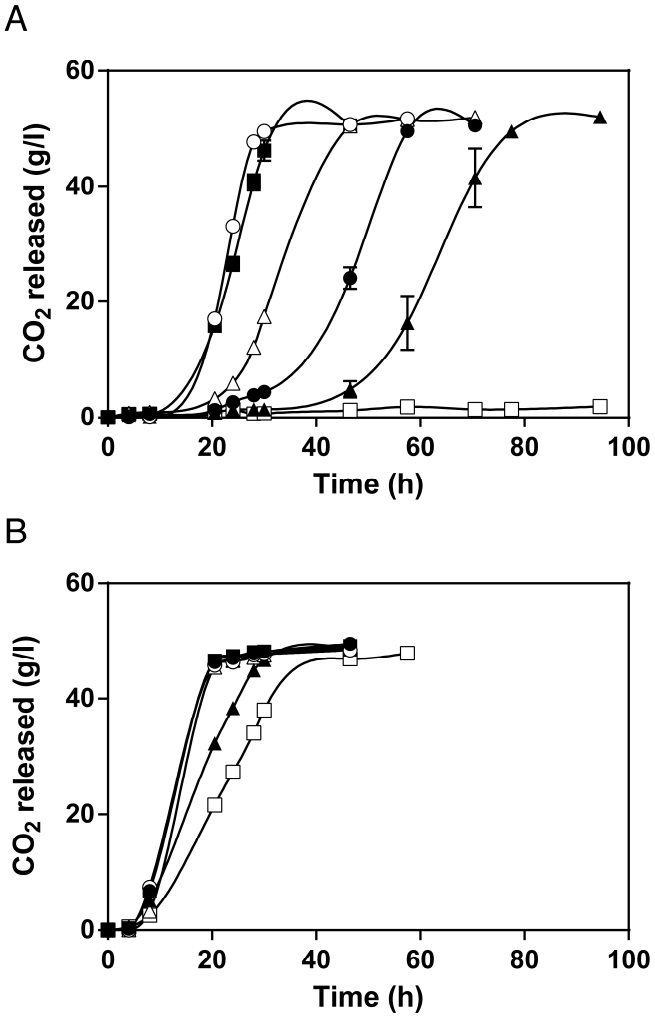


**Fig. 4:** Panel A: Fermentation efficiency of multitolerant yeast strains at 12% (w/v) glucose with inhibitor cocktail containing 1.02 g $l^{-1}$ 5-HMF, 0.33 g $l^{-1}$ furfural, 1.9 g $l^{-1}$ acetic acid, 0.36 g $l^{-1}$ formic acid, 0.7 g $l^{-1}$ levulinic acid and 0.033 g $l^{-1}$ vanillin. Panel B: Fermentation efficiency of multitolerant yeast strains in the absence of inhibitors. CAT 1 (●), Ethanol Red (○), S288c (▲), Y312 (△), Y232 (■), Y1 (□).

**Table 4:** Fermentation performance of multitolerant strains Y232 and Y312, bioethanol strains CAT1 and Ethanol Red and sensitive strains S288c and Y1 in fermentations with 12% glucose (w/v) in the presence and absence of the inhibitor cocktail[(a)].

| | 12% glucose | | | | 12% glucose + Inhibitor | | | |
|---|---|---|---|---|---|---|---|---|
| | Ethanol yield/ 100g of sugar | Theoretical Yield (%) | Time to reach Vmax (h) | C50 (h)[(b)] | Ethanol yield/ 100g of sugar | Theoretical Yield (%) | Time to reach Vmax (h) | C50 (h)[(b)] |
| CAT1 | 49.33 | 96 | 13.07 | 13.35 | 48.58 | 95 | 50.11 | 48.68 |
| Ethanol Red | 49.02 | 96 | 12.49 | 13.35 | 51.48 | 101 | 23.39 | 22.91 |
| Y232 | 50.46 | 99 | 13.07 | 13.35 | 50.17 | 98 | 25.30 | 24.81 |
| Y312 | 50.44 | 99 | 13.65 | 14.52 | 48.29 | 94 | 31.02 | 34.36 |
| S288c | 51.37 | 100 | 14.81 | 17.42 | 49.52 | 97 | 63.48 | 63.95 |
| Y1 | 48.23 | 94 | 17.71 | 23.23 | 2.10 | 4 | 21.48 | NA |

[(a)] 1.02 g $l^{-1}$ 5-HMF, 0.33 g $l^{-1}$ furfural, 1.9 g $l^{-1}$ acetic acid, 0.36 g $l^{-1}$ formic acid, 0.7 g $l^{-1}$ levulinic acid and 0.033 g $l^{-1}$ vanillin

[(b)] C50= Time to consume 50% of the initial sugar content

Pastor et al. 2009; Yoshikawa et al. 2009). Interestingly, the genome expression study of Causton et al. (2001) demonstrated remarkable similarity in yeast response between high salt and high sorbitol concentration and therefore suggested that the change in yeast gene expression under high salt concentration is due to the change in osmotic conditions. However, in our study sugar tolerance poorly correlated with NaCl and LiCl tolerance and only moderately with KCl tolerance. This suggests that tolerance to these solutes might be driven by (partially) different mechanisms. In line with this hypothesis, it has been shown that stress due to high NaCl, LiCl or KCl concentrations is not primarily due to hyperosmotic stress, but rather to the toxicity of high intracellular cation concentrations (Mulet et al. 1999). In line with previous studies (Mulet et al. 1999; Pastor et al. 2009) we also observed that the thresholds of intracellular toxicity for these monovalent cations differ from one another where $Li^+$ has been identified as the most potent stressor followed by $Na^+$ and $K^+$.

In general, *S. pastorianus* was the least tolerant *Saccharomyces* species tested, especially for osmotic, ethanol and 5-HMF stress. This might be partly explained by the fact that *S. pastorianus* is mainly used in and adapted to low temperatures in mild lager beer fermentation conditions (Baerends et al. 2009; Peris et al. 2011; Sanchez et al. 2012), while tests here were performed at 30 °C. Despite having considerably higher genetic diversity, *S. paradoxus* is often reported to have less phenotypic diversity in comparison to its domesticated relative *S. cerevisiae* (Liti et al. 2009; Warringer et al. 2011). Contrary to these studies, the *S. paradoxus* isolates tested in this study (38 isolates) did not show any overall significant difference in phenotypic diversity compared to the *S.*

*cerevisiae* isolates. Differences in the stressors evaluated, the evaluation method and the strain collections studied might explain this discrepancy between the present study and the previous studies. However, when we consider individual traits, we found that for some traits, *S. paradoxus* isolates have significantly lower phenotypic diversity compared to *S. cerevisiae* isolates. However, it has to be emphasized that in this study the *S. cerevisiae* isolates set was considerably larger than that of *S. paradoxus*.

Both *S. paradoxus* and *S. bayanus* have been reported previously as less tolerant to ethanol (Arroyo-López et al. 2010) and less thermotolerant (Gonçalves et al. 2011) compared to *S. cerevisiae*. In agreement with these findings, *S. cerevisiae* performed significantly better than *S. paradoxus* also in this study. However, *S. bayanus* performed as well as *S. cerevisiae* in both ethanol and thermal stress conditions. This might be explained by the fact that *S. bayanus* strains in this collection mainly originated from wine fermentations (seven strains out of ten). This might introduce a bias for the high temperature and especially the ethanol tolerance.

Within the domesticated *S. cerevisiae* isolates, our results showed that ale strains showed significantly higher phenotypic diversity than isolates from other origins (except sake). This may be explained by the pervasive poly- and aneuploidy of ale beer strains (Legras et al. 2007; Querol and Bond 2009), that may confer phenotypic plasticity under different environmental conditions (Comai 2005). The phenotypic diversity of sake strains was as wide as that of ale strains ($p$=0.374). This is caused by the fact that the sake strains investigated are mostly either sensitive or very tolerant to the different environmental conditions assayed in this study, which is in line with the findings of Kvitek et al. (2008).

Within the set of *S. cerevisiae* isolates, phenotypic variability among the non-domesticated *S. cerevisiae* isolates was found to be more or less similar to that of the domesticated *S. cerevisiae*. Interestingly, *S. cerevisiae* strains from wine and bioethanol production as well as the wild isolates investigated, generally showed considerable tolerance to most of the traits (Fig. 3). On the contrary, ale beer strains appeared to be the least tolerant to the conditions tested. Moreover, the performances of wild *S. cerevisiae* isolates were often equally good or sometimes even significantly better compared to the isolates from domesticated origins (wine, sake, bakery, spirit and ale). This suggests that domestication events specific for these origins did not contribute any improvements over these traits during the course of centuries-long evolution. This agrees with the hypothesis that domestication events do not greatly influence the phenotypic diversity among the *S. cerevisiae* population (Liti et al. 2009). Treu et al. (2014) also suggested that domestication events are not always crucial as they showed that many wild vineyard isolates own appropriate phenotypic characteristics for wine fermentation. As an exception, results showed that 73% of wine isolates

were tolerant to 0.1 mM of Cu. Kvitek et al. (2008) suggested that Cu tolerance might have evolved in wine isolates through positive selection as copper is traditionally used in the vineyards and orchards as antimicrobial agent. Warringer et al. (2011) demonstrated a strong association of the sake lineage with a copy number variation of the copper binding metallothionein *CUP1* gene, which could explain the high Cu tolerance of sake strains, a result confirmed in this study. Interestingly, also baker's yeasts were found to be exceptionally tolerant to copper. Since these strains are traditionally not associated with high copper stress, this peculiar phenotype might be explained by the historical origin of baker's yeast. It was recently proposed that most modern baker's yeast strains result from an allotetroploidization event between an ale and a wine yeast (Randez-Gilet al. 2013). Therefore, genetic inheritance of the wine strain's high copper tolerance could explain this phenotype.

In addition to this phenotypic variability analysis this dataset allows selection of multitolerant strains suited for bioethanol production. Not surprisingly currently used bioethanol strains were in general considerably tolerant to multiple stress factors (osmotolerance, ethanol tolerance, thermotolerance, 5-HMF tolerance). However, we also identified multitolerant isolates from other origins that sometimes even outperformed the bioethanol strains for certain traits. Therefore, we hypothesized that these multitolerant strains coming from other origins might have great potential for commercial bioethanol production. In order to validate this hypothesis two proof-of-principle fermentation experiments were conducted. The first experiment at 33% glucose indicated that in terms of ethanol yield, all the tested multitolerant strains performed nearly as well as industrial strain CAT1. However, Ethanol Red outcompeted all other strains and was clearly superior in conditions mimicking first-generation bioethanol production. Nevertheless, in a second fermentation mimicking the harsh conditions in lignocellulose hydrolysates by addition of fermentation inhibitors, the strain Y232 (originating from wine) showed to be as efficient as Ethanol Red and better than CAT1. The wild strain Y312 also outperformed CAT1.

Together, this large-scale and high-throughput phenotypic analysis yielded a multi-purpose database, providing novel insights in general inter- and intra-species phenotypic diversity of *Saccharomyces* yeasts. Additionally, it provides a basis for new insights into the relationships between tolerance to different stressors. Our results also illustrate the potential of phenotypically evaluating the natural biodiversity of yeasts to find superior industrial strains that may be used in bioethanol production. In addition, it may provide excellent candidates for further strain improvement through genetic engineering, experimental evolution, or breeding.

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